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A Diclofop-methyl-Resistant *Avena sterilis* Biotype with a Herbicide-Resistant Acetyl-coenzyme A Carboxylase and Enhanced Metabolism of Diclofop-methyl

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Abstract: An *Avena sterilis* biotype was found to be highly resistant to aryloxyphenoxypropionate (APP) herbicides, especially diclofop-methyl. At the enzyme level, this biotype contained a modified acetyl-coenzyme A carboxylase (ACCase) with six-fold resistance to diclofop acid. Absorption and translocation of [^{14}C]diclofop-methyl applied to the leaf axil of the two-leaf stage plants were similar in both susceptible and resistant biotypes. However, the rate of metabolism of [^{14}C]diclofop was increased 1·5-fold in this resistant biotype compared to the susceptible. Experiments with tetcyclacis, a cytochrome P450 monooxygenase inhibitor, indicated that inhibition of this enhanced diclofop metabolism increased diclofop-methyl phytotoxicity in this biotype. Studies with ten individual families of the resistant biotype indicated that both mechanisms of resistance, an altered target site and enhanced metabolism, are present in each individual of the population. Hence, it is likely that these two mechanisms of resistance both contribute to resistance in this biotype.

Key words: acetyl-coenzyme A carboxylase, *Avena sterilis*, diclofop-methyl, metabolism, tetcyclacis

1 INTRODUCTION

Diclofop-methyl is an aryloxyphenoxypropionate (APP) herbicide widely used in Australia for controlling grass weeds, mainly *Avena* spp. (wild oat) and *Lolium rigidum* Gaud. (annual ryegrass), in cereal crops. Since the late 1980s many *Avena* spp. populations exhibiting resistance to diclofop-methyl have appeared following exposure to this and other APP herbicides.[1] Two classes of herbicides, the APP and cyclohexanedione (CHD) herbicides, are structurally different but have the same mode of action. These herbicides are potent inhibitors of acetyl-coenzyme A carboxylase (ACCase, EC 6.4.1.2), a key enzyme in fatty acid biosynthesis,[2] and also depolarise the plasma membrane potentials of susceptible grass species.[3] Most dicotyledonous species are tolerant of diclofop, due to possession of an insensitive ACCase, whereas the ACCase from grass species is susceptible.[4,5] Although wheat contains a sensitive ACCase, diclofop is rapidly metabolised by cytochrome P450 monooxygenases into non-toxic products in this species.[6,7]

Recently, resistance to diclofop-methyl has been documented in several weed species such as biotypes of *L. rigidum*, *L. multiflorum* Lam., *Eleusine indica* (L.) Gaertn., *Setaria viridis* (L.) Beauv. and *Avena* spp.[1,8–12] A target-site-based mechanism, an insensitive ACCase, is the most common resistance mechanism reported in these species. However, other mechanisms, such as enhanced metabolism of diclofop, are also possible, as has been documented in some *L. rigidum* biotypes. For example, *L. rigidum* biotype SLR 31 was able to detox-

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ify diclofop acid at about 1·5 times of the rate of the susceptible biotype.[13] To date, enhanced metabolism of diclofop has not been observed in biotypes of *Avena* spp. In addition, resistance to ACCase-inhibiting herbicides has been correlated with the ability of the plasma membrane potential to recover from diclofop-induced depolarisation in several biotypes of *L. rigidum*[3,13,14] and *A. fatua* biotype UM-1.[15]

Two species of wild oat, *A fatua* L. and *A. sterilis* L. commonly infest cereal fields in southern Australia.[16] The only morphological feature that distinguishes *A. fatua* from *A. sterilis* is that the second floret disarticulates readily in the former, but not the latter;[17] however, natural hybrids between *A. fatua* and *A. sterilis* may occur.[18,19] In southern Australia, both species germinate in autumn to early winter, set seed between September and December,[17] and can occur in the same field (Nietschke, pers. comm.). Herbicide resistance has developed in populations of both *A. fatua* and *A. sterilis* in Australia.[1] A biotype of *A. sterilis*, designated NAS 4, developed resistance to diclofop-methyl after eight applications of APP and two applications of CHD herbicides. This biotype also proved resistant to other APP herbicides. Possible mechanisms conferring resistance in this biotype, such as an ACCase insensitive to APP and CHD herbicides, reduced absorption and translocation, enhanced metabolism of [^{14}C]diclofop-methyl, as well as the ability of plasma membrane to recover from diclofop-induced membrane depolarisation by diclofop acid were investigated.

2 MATERIALS AND METHODS

2.1 Dose response to herbicides

Resistant *A. sterilis* biotype NAS 4 was collected from a field in New South Wales which had been exposed to eight applications of APP and two applications of CHD herbicides during 11 years before developing resistance to ACCase-inhibiting herbicides. Susceptible *A. fatua* biotype SAF 19 was collected from a field area in South Australia with no previous recorded use of herbicides. This susceptible *A. fatua* biotype is highly sensitive to all APP and CHD herbicides with a similar dose-response to susceptible biotypes of *A. sterilis* and *A. fatua* reported earlier.[1,12] In some experiments, a second resistant *A. sterilis* biotype,[1,12] designated SAS 1 was used. This biotype is highly resistant to all APP herbicides due to a highly resistant form of ACCase, but rates of absorption, translocation and metabolism of diclofop-methyl are identical to those of susceptible biotypes.[12]

Plants were sprayed at the two- to three-leaf stage, the most susceptible stage under agricultural conditions. The following herbicide formulations were used: diclofop-methyl 375 g litre^{-1} EC ('Hoegrass'; Hoechst Schering AgrEvo), fluazifop-butyl 212 g AE litre^{-1} EC ('Fusilade'; Crop Care Australia Pty.), haloxyfop-etotyl 104 g AE litre^{-1} EC ('Verdict'; DowElanco) and tralkoxydim 100 g litre^{-1} EC ('Grasp'; Crop Care Australia Pty.). These were applied as dilutions in tap water with an additional 2 g litre^{-1} nonionic surfactant in a laboratory spray cabinet and delivered *via* two 110° flat-fan hydraulic nozzles suspended 40 cm above the plant axils. At a pressure of 250 kPa and a boom speed of 1 m s^{-1}, the sprayer output was 113 litre ha^{-1}. Herbicides were applied at rates that ranged from 0·125 to 64 times the recommended field rates of the herbicides used.

Six plants of each biotype were grown in each pot. Within each herbicide treatment, pots were arranged as a completely randomised block design with six replicates. Plants were harvested four weeks after spraying which allowed surviving plants to make noticeable growth. Plants which were green, had live meristems and had increased in size beyond the two-leaf stage were classified as live plants.

2.2 Measurement of ACCase activity

ACCase was extracted from the shoot meristematic region of two- to three-leaf plants of susceptible SAF 19 and resistant NAS 4 biotypes, partially purified and assayed in the presence of herbicides (diclofop acid, fluazifop acid, haloxyfop acid, sethoxydim and tralkoxydim) as previously described.[12]

2.3 Absorption and translocation of [^{14}C]diclofop-methyl

Germinated seedlings of susceptible SAF 19 and resistant NAS 4 biotypes were transferred to 1-litre rectangular plastic containers containing 500 ml of nutrient solution.[20] Containers were wrapped with aluminium foil to prevent light reaching the roots. Seedlings were supported by black polypropylene beads (400 ml per container) then transferred to the growth chamber and were maintained at 20°C, 14 h, 300 μmol photons m^{-2} s^{-1} light period/16°C, 10 h dark period. The containers were topped up with about 100 ml water every day to replace evapo-transpirational losses in the first week. Afterwards, half-strength nutrient solution was added until the end of the experiments.

At the two-leaf stage, plants were treated with 1 μl of 5 mM diclofop-methyl (4·5 mM [^{14}C]diclofop-methyl, 592 Bq, dissolved in diclofop-methyl EC equivalent to 0·5 mM AI) onto the leaf axil. Five plants of each biotype (one plant per replicate) were harvested 1, 3, 6, 12, 24, 48 and 96 h after treatment.

Unabsorbed radioactivity was removed by washing the plant in 5 ml of water + methanol + 'Triton'

X-100 (3·995 + 1 + 0·005 by volume). Radioactivity in the washes was measured by liquid scintillation spectroscopy (LSS) using an water-compatible scintillant (Ultima Gold, Packard, Tampa, FL, USA). The plant was sectioned into four fractions: shoot meristematic region (1 cm above the initiation root zone), stem (from meristematic zone up to 2 cm above the leaf axil), leaves and roots. Plant parts were combusted in a Biological Sample Oxidizer (R.J. Harvey Corporation, Milldale, NY, USA), the evolved [^{14}C]carbon dioxide trapped in 14 ml of carbon trap and scintillation cocktail (Carbosorb + Permafluor E^+, 1 + 1 by volume; Canberra-Packard, Tampa, FL, USA), and the radioactivity quantified by LSS.

2.4 Metabolism of [^{14}C]diclofop-methyl

Two-leaf stage seedlings of susceptible SAF 19 and resistant NAS 4 biotypes grown in soil were treated with 1 μl of 5 mM [^{14}C]diclofop-methyl on to the leaf axil as described above. Treated plants were harvested 1, 3, 6, 12, 24, 48 and 72 h after herbicide application. Radiolabelled metabolites were extracted and identified by HPLC as described previously.[12] Twelve plants of each biotype were used as a replicate.

To demonstrate the effects of tetcyclacis on the metabolism of diclofop-methyl, susceptible SAF 19 and resistant NAS 4 biotypes were grown hydroponically under growth-room conditions as described for uptake and translocation experiments. (Section 2.3) Tetcyclacis (20 μM) was added to the nutrient solution 24 h prior to treatment with 1 μl of 5 mM [^{14}C]diclofop-methyl applied to the leaf axil. Plants were harvested 24 h following herbicide treatment, ground, radioactivity extracted and the metabolites separated by HPLC. The experiments were designed as a completely randomised block with four replicates. Twelve plants of each biotype were used as a replicate.

2.5 Effect of tetcyclacis and diclofop-methyl on plant growth

To determine the effects of tetcyclacis on the growth of susceptible SAF 19 and resistant NAS 4 biotypes, these were cultured hydroponically in 1-litre rectangular plastic trays. Each tray contained a single row of each biotype (six plants per row). The nutrient solution was replaced with solution containing 20 μM tetcyclacis when plants were at the two-leaf stage. After treatment with 20 μM tetcyclacis for 24 h, a 1 μl droplet of 0, 2·5, 5 or 10 mM diclofop-methyl (commercial formulation) was placed on the leaf axil of two-leaf stage plants. Seven days after herbicide application, dry weights of shoot and root of individual plants were measured. The experiments were designed as a completely randomised block with four replicates.

2.6 Inhibition of ACCase by diclofop in single lines of NAS 4

To test the hypothesis that two mechanisms of resistance to diclofop are present in each individual of the resistant NAS 4 population, seeds from ten different plants of this resistant biotype were collected separately. Germinated seedlings were transplanted into sterile potting mix based on sand + peat (1 + 1 by volume) in 15-cm pots. At the two- to three-leaf stage, shoot meristematic tissue was harvested and the ACCase extracted. The extracted ACCase was assayed for activity in the presence and absence of diclofop acid. Two lines of NAS 4, and one each of resistant biotype SAS 1 and susceptible biotype SAF 19 were arranged in each assay with four replicates.

2.7 Plasma membrane potential measurements

Plasma membrane potentials of etiolated coleoptiles from susceptible SAF 19 and resistant NAS 4 biotypes were measured as described previously.[12] Ten coleoptiles of susceptible SAF 19 and resistant NAS 4 seedlings were used and the data shown represent a typical result for each biotype. Potentials were initially measured in Higinbotham's solution[21] and, when a stable reading had been achieved, this solution was replaced by a solution supplemented with 50 μM diclofop acid. Fifteen minutes after addition of diclofop acid the solution was replaced by herbicide-free solution.

3 RESULTS

3.1 Response to herbicides at the whole plant and enzyme levels

When pot-grown plants were treated with diclofop-methyl, the susceptible *A. fatua* biotype SAF 19 was readily controlled by the herbicide. The normal use rate of 500 g ha^{-1} killed more than 90% of this biotype. In contrast, the resistant *A. sativa* biotype NAS 4 showed a very high level of resistance to diclofop-methyl and the highest rate of diclofop-methyl used (32 000 g ha^{-1}) resulted in no mortality (Fig. 1). The LD_{50} (concentration of herbicide required to kill 50% of a population) for diclofop-methyl was 167 g ha^{-1} for the susceptible biotype compared to $>$32 000 g ha^{-1} for the resistant biotype. The resistant NAS 4 biotype was also resistant to other APP herbicides and displayed 37- and 10-fold resistance to fluazifop-butyl and haloxyfop-etotyl respectively (Table 1). LD_{50} values for the susceptible SAF 19 biotype for diclofop-methyl, haloxyfop-etotyl, fluazifop-butyl and tralkoxydim were nearly identical to those reported previously for other susceptible biotypes of *A sterilis* and *A. fatua*.[1]

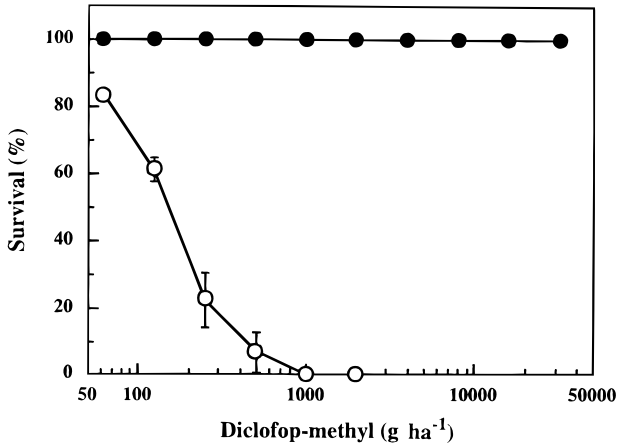

Fig. 1. Survival of (○) susceptible *Avena fatua* biotype SAF 19 and (●) resistant *Avena sterilis* biotype NAS 4 30 days after exposure to diclofop-methyl. Each point is the mean ± SE of six replicates.

The target site for these herbicides, ACCase, was extracted from the resistant NAS 4 biotype and proved to be less sensitive to inhibition by these ACCase-inhibiting herbicides than that of the susceptible SAF 19 biotype. The ACCase extracted from the resistant NAS 4 biotype was six-, 24- and nine-fold less sensitive to diclofop acid, fluazifop acid and haloxyfop acid, respectively (Table 1). The resistant NAS 4 biotype displayed low-level resistance to the cyclohexanedione herbicide, tralkoxydim (Table 1), which was reflected at the enzyme level. With the exception of diclofop, there was a good correlation between resistance in biotype NAS 4 at the whole plant and enzyme levels. Clearly, resistance to most ACCase-inhibiting herbicides in biotype NAS 4 is the result of possession of a herbicide-insensitive ACCase. Nevertheless, when compared with another resistant *A. sterilis* biotype SAS 1,[1] the resistant NAS 4 biotype tested here is more resistant to diclofop-methyl. Biotype SAS 1 possesses an ACCase with 52-fold resistance to diclofop acid,[12] whereas that of biotype NAS 4 is only six-fold resistant. Therefore, the modified target site, ACCase, in biotype NAS 4 may be insufficient to confer the high level of resistance to diclofop-methyl observed in whole plants. To test this hypothesis, other possible mechanisms of resistance were examined.

3.2 Absorption and translocation of [^{14}C]diclofop-methyl

Absorption and translocation of diclofop-methyl were determined following application of 1 μl of 5 mM [^{14}C]diclofop-methyl to the leaf axil of the two-leaf seedlings. Radioactivity from diclofop-methyl was absorbed rapidly following application (Fig. 2) with no differences between the two biotypes. Translocation of ^{14}C from the treated area to the meristematic region was similar for both biotypes and reached maxima of 12% and 10% in the susceptible SAF 19 and resistant NAS 4 biotypes, respectively (Fig. 3(a)). More than 85% of the absorbed herbicide was observed in the stem

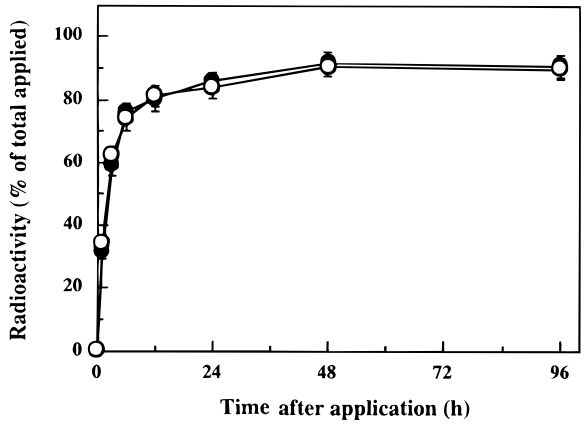

Fig. 2. Absorption of [^{14}C]diclofop-methyl by (○) susceptible *Avena fatua* biotype SAF 19 and (●) resistant *Avena sterilis* biotype NAS 4. [^{14}C]diclofop-methyl was applied to the leaf axil of two-leaf stage seedlings. Points are the mean ± SE of five replicate experiments.

TABLE 1

Amounts of APP and CHD Herbicides giving 50% Mortality (LD_{50}) and 50% Inhibition of ACCase Activity (I_{50}) from the Susceptible *Avena fatua* Biotype SAF 19 (S) and Resistant *Avena sterilis* Biotype NAS 4 (R). APP esters were applied to intact plants and acids were used in ACCase assays

	LD_{50} (g ha^{-1})[a]			I_{50} (μM)[a]		
Herbicide	*R*	*S*	*R/S*[b]	*R*	*S*	*R/S*[b]
Aryloxyphenoxypropionate herbicides						
Diclofop-methyl/diclofop acid	>32 000[c]	167	⩾192	6·3	1·1	6
Fluazifop-butyl/fluazifop acid	448	12	37	78	3·2	24
Haloxyfop-etotyl/haloxyfop acid	80	8	10	16	1·8	9
Cyclohexanedione herbicides						
Sethoxydim	—	—	—	14	5	3
Tralkoxydim	36	20	2	1·7	0·8	2

[a] LD_{50} and I_{50} values calculated from pooled data of six and four replicates, respectively.
[b] Value for the resistant NAS 4 divided by the value for the susceptible SAF 19 biotype.
[c] No mortality of the resistant biotype occurred at 32 000 g ha^{-1} diclofop-methyl.

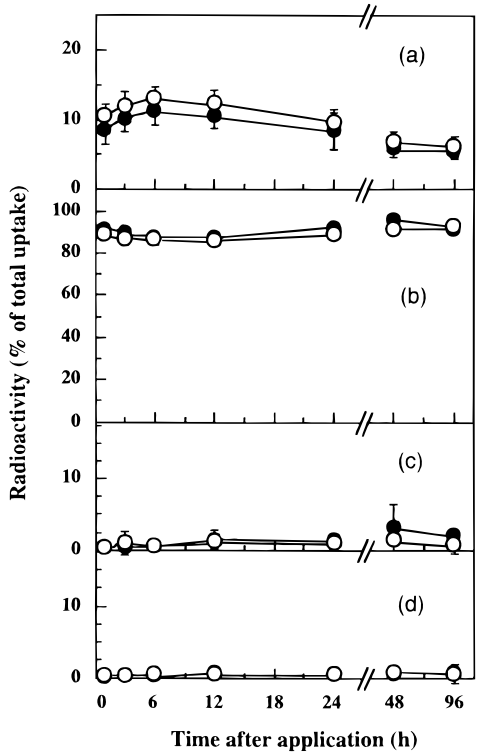

Fig. 3. Distribution of radioactivity in (a) the meristem, (b) stem, (c) leaf and (d) root from [^{14}C]diclofop-methyl deposited on the leaf axil of two-leaf stage seedlings of (○) susceptible *Avena fatua* biotype SAF 19 and (●) resistant *Avena sterilis* biotype NAS 4. Points are the mean ± SE of five replicate experiments.

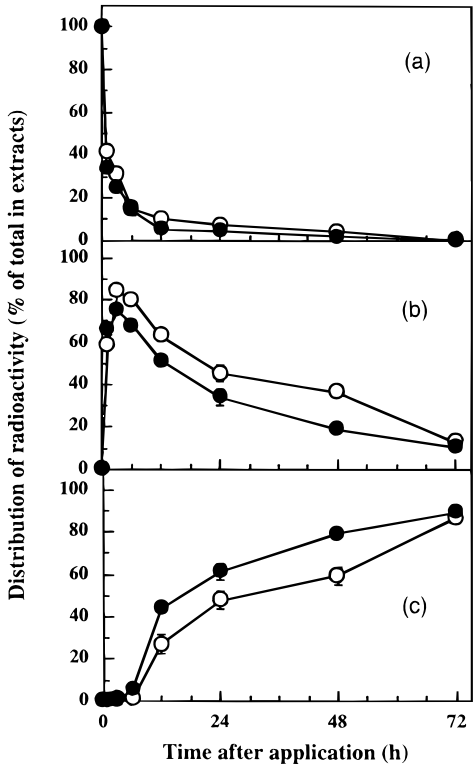

Fig. 4. Distribution of radioactivity as (a) diclofop-methyl, (b) diclofop acid or (c) other metabolites up to 72 h after treatment with 1 μl of 5 mM [^{14}C]diclofop-methyl to (○) susceptible *Avena fatua* biotype SAF 19 and (●) resistant *Avena sterilis* biotype NAS 4. Points are the mean ± SE of three replicates.

region up to 96 h following application of herbicide (Fig. 3(b)). Very little radioactivity was translocated to the leaf tips and roots of either biotype (Fig. 3(c), (d)). Therefore, differences in absorption and translocation do not contribute to resistance to diclofop in biotype NAS 4.

3.3 Metabolism of [^{14}C]diclofop-methyl

Metabolism of diclofop-methyl was determined in experiments similar to those used for absorption and translocation. In susceptible SAF 19 and resistant NAS 4 biotypes, [^{14}C]diclofop-methyl was degraded to diclofop acid at an equal rate (Fig. 4(a), (b)). Radioactivity rapidly accumulated in the phytotoxic form, diclofop acid, with about 60% of the label in this form 1 h after treatment (Fig. 4(b)). Conversion of diclofop acid to the other metabolites was more rapid in the resistant NAS 4 biotype than in the susceptible SAF 19 (Fig. 4(c)). From 6 to 48 h after treatment, the susceptible SAF 19 tissue contained between 5 and 15% more of the radiolabel as [^{14}C]diclofop acid than did the resistant NAS 4 tissue. The decline in diclofop acid was accompanied by an increase in more polar metabolites in both biotypes. These metabolites were first evident at 6 h after treatment in the resistant NAS 4 biotype and were produced more rapidly by this biotype (Fig. 4(c)). By 72 h after treatment, the contents of [^{14}C]diclofop acid had decreased to 14 and 11% in susceptible SAF 19 and resistant NAS 4 biotypes, respectively (Fig. 4(b)), with no differences between the two biotypes. However, the initial rate of production of polar metabolites was about 1·5 times faster in the resistant NAS 4 biotype than in the susceptible SAF 19 biotype (Fig. 4(c)). The rates of de-esterification of diclofop-methyl and further metabolism of diclofop acid in the susceptible SAF 19 biotype were similar to those reported earlier for a susceptible *A. sterilis* biotype.[12] The HPLC elution profiles for the resistant NAS 4 and susceptible SAF 19 biotypes at 24 h after treatment were similar except that the resistant biotype had more polar metabolites eluting between 4 and 7 min than did the susceptible (Fig. 5(a), (b)).

It is clear that the resistant NAS 4 biotype is able to convert diclofop acid to more polar compounds at a faster rate than the susceptible SAF 19 biotype. There are two reactions which may be involved in this conversion. In wheat, diclofop is predominantly metabolised *via* an aryl-hydroxylation reaction catalysed by a cytochrome P450 monooxygenase.[7,22] The arylhydroxylated diclofop is then conjugated to sugars and other compounds.[23] In some susceptible grass species, diclofop is predominantly metabolised *via* an ester linkage to a glucose moiety.[24]

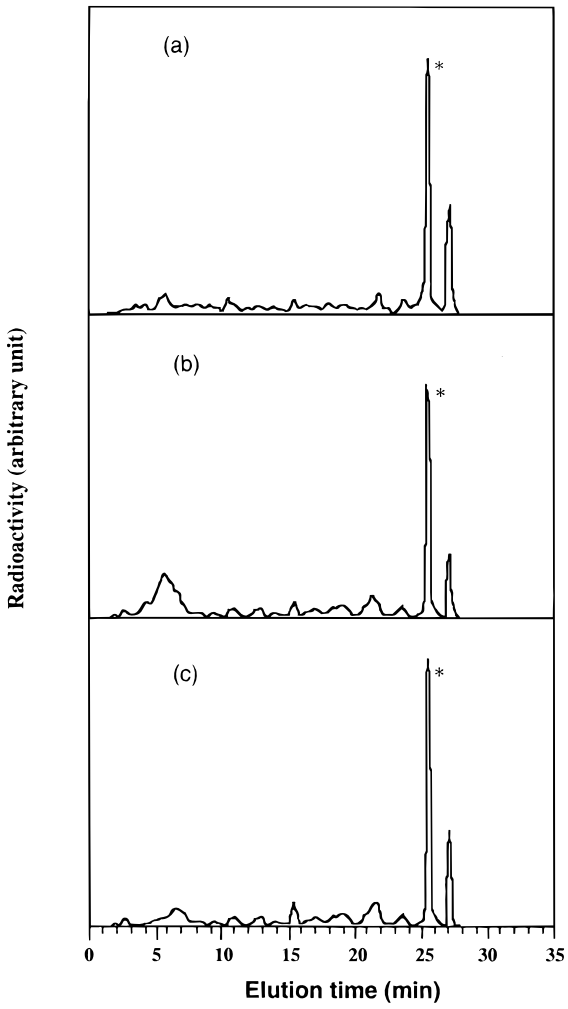

Fig. 5. HPLC elution profiles of extracts from (a) susceptible *Avena fatua* biotype SAF 19 and (b) resistant *Avena sterilis* biotype NAS 4 24 h after exposure to [^{14}C]diclofop-methyl dissolved in a commercial herbicide formulation. (c) HPLC elution profile of an extract from the resistant NAS 4 biotype pre-treated with 20 μM tetcyclacis 24 h prior to application of the [^{14}C]diclofop-methyl. [^{14}C]Diclofop acid eluted with a retention time of 26 min (*).

To test for involvement of cytochrome P450 enzymes in metabolism of diclofop in the resistant wild oat biotype, tetcyclacis, a broad-spectrum cytochrome P450 inhibitor,[25–27] was applied in combination with diclofop. Tetcyclacis was applied 24 h before application of diclofop-methyl to wild oat plants. By 24 h following application of diclofop-methyl alone, the susceptible SAF 19 biotype contained about 12% more of the extractable radioactivity as diclofop acid than did the resistant NAS 4 biotype (Table 2). Pre-treatment with tetcyclacis did not affect metabolism of diclofop acid in the susceptible SAF 19 biotype. However, pre-treatment of the resistant NAS 4 biotype with tetcyclacis reduced metabolism of diclofop acid to a level similar to that of the susceptible SAF 19 biotype. In addition, tetcyclacis treatment of the resistant NAS 4 biotype preferentially inhibited production of metabolites eluting between 4 and 7 min (Fig. 5(c)), thereby eliminating differences in metabolite profiles between the two biotypes.

3.4 Effects of tetcyclacis on plant growth

To examine the effects of tetcyclacis on growth of intact plants, susceptible SAF 19 and resistant NAS 4 biotypes exposed to 0 or 20 μM tetcyclacis were treated with different concentrations of diclofop-methyl. Tetcyclacis alone reduced dry weight of susceptible SAF 19 by 16% and resistant NAS 4 by 9%. Diclofop-methyl applied at all concentrations did not affect total dry weight of plants of the resistant NAS 4 biotype. Treatments as low as 2·5 mM reduced dry weight of the susceptible SAF 19 biotype to about 40% of that of the control (Fig. 6) and at 10 mM reduced dry weight of this biotype to less than 20% of that of the control. Application of tetcyclacis in combination with diclofop-methyl did not decrease total dry weight of the susceptible SAF 19 biotype by more than the reduction due to diclofop-methyl applied alone. Therefore there was no observed synergistic effect of these two compounds in the susceptible SAF 19 biotype. In contrast, tetcyclacis applied in combination with diclofop-methyl had a synergistic effect on total dry weight of the resistant NAS 4 biotype. At 10 mM diclofop-methyl in the presence of tetcyclacis, total dry weight was reduced by 40%, compared to no reduction in the absence of tetcyclacis (Fig. 6).

TABLE 2
Amount of Radiolabel as [^{14}C]Diclofop Acid in the Susceptible *Avena fatua* Biotype SAF 19 and Resistant *Avena sterilis* Biotype NAS 4 24 h after Treatment with 5 mM [^{14}C]Diclofop-methyl with or without 20 μM Tetcyclacis. Tetcyclacis was Applied 24 h prior to Application of Herbicide

Treatment	*Biotype*	*Diclofop acid* (%) ($\pm SE$)[a]
Diclofop-methyl	S	41·8 (±1·5)
	R	30·1 (±1·3)
Diclofop-methyl + tetcyclacis	S	41·1 (±1·6)
	R	39·1 (±1·7)

[a] $n = 5$.

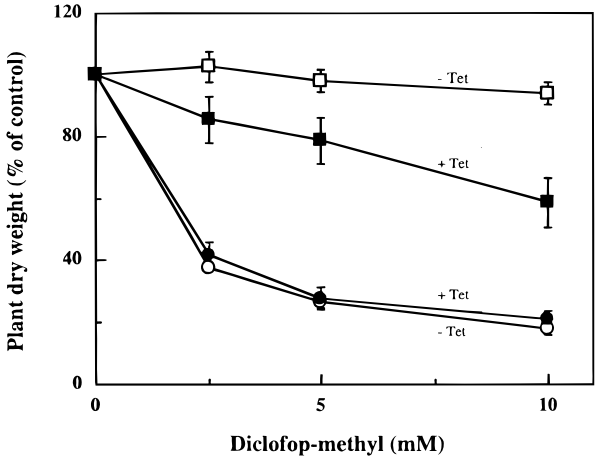

Fig. 6. Total dry weight of (●, ○) susceptible *Avena fatua* biotype SAF 19 and (■, □) resistant *Avena sterilis* biotype NAS 4 biotypes grown in hydroponic culture when treated with (open symbols) diclofop-methyl alone or (closed symbols) in the presence of 20 μM tetcyclacis (Tet). Control values were 147·1(±7·29), 123·8(±10·2), 152·4(±5·2) and 139·2(±8·3) mg per plant for untreated susceptible, tetcyclacis-treated susceptible, untreated resistant and tetcyclacis-treated resistant plants, respectively. Point symbols are the mean ± SE of four replicates.

These experiments demonstrate that enhanced diclofop metabolism contributes to diclofop-methyl resistance in the resistant NAS 4 biotype. Tetcyclacis inhibits metabolism of diclofop acid in this biotype and enhances the phytotoxicity of diclofop-methyl applied to intact plants. Tetcyclacis did not increase phytotoxicity of diclofop, as determined by plant dry weight, in the susceptible SAF 19 biotype where it does not decrease diclofop metabolism. The resistant NAS 4 biotype did not become fully susceptible upon treatment with tetcyclacis, probably because this biotype also contains a diclofop-resistant ACCase.

3.5 Inhibition of ACCase activity by diclofop acid in single lines of biotype NAS 4

The resistant NAS 4 biotype has two mechanisms of resistance to diclofop-methyl, a less-sensitive ACCase (Table 1) and enhanced metabolism of diclofop (Fig. 4). To establish whether both mechanisms are present within all individuals of the population, seed of ten individuals was collected and each family tested for the presence of a diclofop-resistant ACCase. These lines were compared to the susceptible SAF 19 biotype with a sensitive ACCase and another resistant biotype, SAS 1, with a highly resistant ACCase. ACCase from the resistant biotype SAS 1 exhibited 50-fold less sensitivity to diclofop inhibition than that of susceptible SAF 19 biotype. This level of resistance is comparable to that reported previously for this biotype.[12] Concentrations of diclofop acid giving 50% inhibition (I_{50}) of ACCase activity from the ten lines of biotype NAS 4 ranged from 1·2 to 3·0 μM. The I_{50} values of ACCase from these NAS 4 lines were between six and 15 times greater than that of the susceptible SAF 19 biotype (Table 3). This corresponds well with the ACCase extracted from the whole population of NAS 4 biotype which was found to be six-fold more resistant to diclofop than the susceptible SAF 19 biotype (Table 1). These small differences of R/S values between the single lines and bulk population suggest that the population of the resistant NAS 4 biotype is homogeneous and all individuals contain a resistant ACCase. The six- to 15-fold resistance to diclofop in the single lines and in the bulk population of the resistant NAS 4 biotype is probably insufficient to account for the high degree of resistance to diclofop-methyl at the whole plant level. That is, the resistant NAS 4 biotype shows greater resistance to diclofop-methyl than biotype SAS 1, despite possessing an ACCase three- to eight-fold less resistant. Therefore, it is likely that resistance to diclofop-methyl in biotype NAS 4 involves two mechanisms, a mutant ACCase enzyme and enhanced metabolism to detoxify the herbicide, and that these two mechanisms of resistance coexist in all individuals.

3.6 Response of plasma membrane electrogenic potential to diclofop acid

It has been observed previously that diclofop-methyl-resistant biotypes of *L. rigidum*[3,13,14] and *A. fatua*[15] were able to recover from diclofop-acid-induced depolarisation of the plasma membrane potential, whereas

TABLE 3
Concentration of Diclofop Acid giving 50% Inhibition of ACCase Activity (I_{50}) from the Susceptible *Avena fatua* Biotype SAF 19, Resistant *Avena sterilis* Biotype SAS 1 and 10 Lines of Resistant *A. sterilis* Biotype NAS 4 (1–10)[a]

Biotype	$I_{50\ (\mu M)}$ (±*SE*)[b]	*R/S Ratio*[c]
Susceptible SAF 19	0·20 (±0·04)	—
Resistant SAS 1	10·0 (±1·1)	50
Resistant NAS 4 lines		
1	2·5 (±0·5)	13
2	3·0 (±0·7)	15
3	2·1 (±0·6)	11
4	2·0 (±0·5)	10
5	1·4 (±0·6)	7
6	1·3 (±0·5)	7
7	1·2 (±0·6)	6
8	2·0 (±0·4)	10
9	1·8 (±0·6)	9
10	2·1 (±0·6)	11
Mean (1–10)	1·9 (±0·2)	10 ± 1

[a] ACCase activity was measured in partially purified extracts of the shoot meristematic regions.
[b] $n = 4$.
[c] Value for the resistant (either NAS 4 of SAS 1) divided by the value for the susceptible SAF 19.

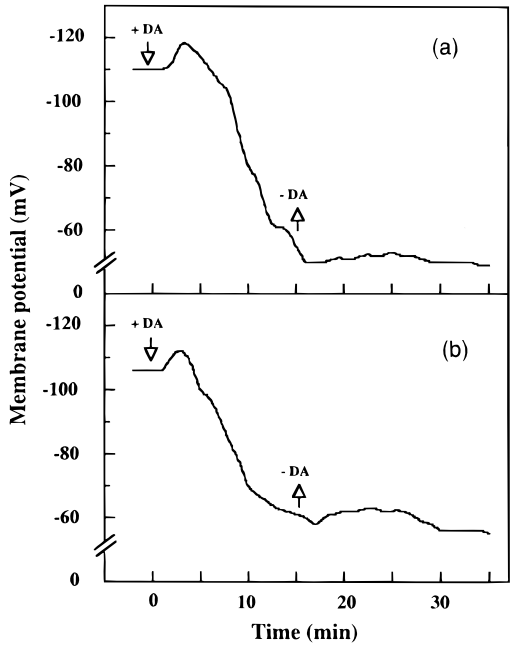

Fig. 7. The effect of 50 μM diclofop acid on plasma membrane potentials in coleoptile cells of (a) the susceptible *Avena fatua* biotype SAF 19 and (b) resistant *Avena sterilis* biotype NAS 4. Arrows, addition (+) and removal (−) of diclofop acid (DA). The data shown are from a single coleoptile and are representative of 10 coleoptiles of biotype.

susceptible biotypes could not. The ability of diclofop acid to depolarise plasma membrane potentials in etiolated coleoptile cells of susceptible SAF 19 and resistant NAS 4 biotypes was examined. Plasma membrane potentials of untreated coleoptile cells from susceptible SAF 19 and resistant NAS 4 biotypes were between −110 and −115 mV (Fig. 7). Diclofop acid (50 μM) depolarised plasma membrane potentials from both biotypes within 15 min to between −50 and −60 mV (Fig. 7(a), (b)). After removal of herbicide, plasma membrane potentials from both biotypes stayed depolarised for at least 20 min. Neither biotype was able to repolarise the plasma membrane potential following diclofop-acid-induced depolarisation. Therefore, changes in plasma membrane properties do not contribute to diclofop-methyl resistance in biotype NAS 4.

4 DISCUSSION

Resistant *A. sterilis* biotype NAS 4 shows high-level resistance to diclofop-methyl, but possesses only a moderately resistant ACCase (Table 1). There were no differences in absorption or translocation of diclofop-methyl in this resistant NAS 4 biotype compared to the susceptible SAF 19 biotype (Figs 2 and 3). Nor does this biotype possess a mechanism which allows rapid repolarisation of the plasma membrane potential following diclofop-acid-induced depolarisation (Fig. 7). However, *in vitro*, the resistant NAS 4 biotype possesses an insensitive ACCase with about six-, 24- and nine-fold resistance to diclofop, fluazifop and haloxyfop, respectively (Table 1). The ACCase from this biotype also shows slight, two- to three-fold, resistance to the CHD herbicides, sethoxydim and tralkoxydim, which correlates with the two-fold resistance to tralkoxydim of whole plants.

In diclofop-resistant weed biotypes possessing a resistant ACCase, there is often a good correlation between resistance *in vivo* and *in vitro*. For example, a resistant biotype of *L. rigidum* with greater than 55-fold resistance to diclofop-methyl contains an ACCase with 37-fold resistance to diclofop acid.[28] Also, *A. sterilis* biotype SAS 1, which displays greater than 150-fold resistance at the whole-plant level, possesses an ACCase with 52-fold resistance to diclofop acid.[12] Similarly, a resistant biotype of *L. multiflorum* with 130-fold resistance to diclofop-methyl contains an ACCase with 28-fold resistance.[10] In comparison, the six-fold resistance to diclofop acid at the enzyme level in biotype NAS 4 appears insufficient to account for the $\geqslant$192-fold resistance to diclofop-methyl at the whole-plant level. In addition to a resistant ACCase, biotype NAS 4 exhibits an increased rate of diclofop acid metabolism (Fig. 4(a)–(c)). A similar result was reported for *L. rigidum* biotype SLR 31 which exhibited a 1·5-fold increase in metabolism of diclofop acid.[13] At the whole-plant level, diclofop-methyl phytotoxicity on the resistant NAS 4 biotype was increased in the presence of the cytochrome P450 inhibitor, tetcyclacis, whereas tetcyclacis had little effect on the response of the susceptible SAF 19 biotype to diclofop-methyl (Fig. 6). The rate of metabolism of diclofop acid in resistant NAS 4 biotype decreased in the presence of tetcyclacis. When plants were treated with tetcyclacis 24 h prior to application of [^{14}C]diclofop-methyl, metabolism of diclofop in the resistant NAS 4 biotype was reduced to the level in the susceptible SAF 19 biotype (Table 2, Fig. 5). These results suggest that the enhanced metabolism of diclofop-methyl observed in biotype NAS 4 is probably due to a cytochrome P450 monooxygenase.

Wheat is resistant to a number of selective herbicides, including diclofop-methyl, due to its ability to metabolise these herbicides rapidly.[5,25,29–31] Wheat contains one or more cytochrome P450 monooxygenases which are responsible for initial steps in metabolism of many herbicides, including diclofop-methyl.[6,7,25,27,32] The laurate hydroxylase in wheat is believed to be the enzyme that is responsible for aryl hydroxylation of diclofop acid.[33] The enzyme responsible for the enhanced diclofop metabolism in the resistant NAS 4 does not appear to be present, or is present with only low activity, in the susceptible SAF 19 biotype, based on the ability of tetcyclacis to inhibit diclofop metabolism. Whether the enzyme(s) responsible for enhanced metabolism of diclofop in the resistant NAS 4 biotype are the same as those in wheat remains to be determined.

The resistance mechanism most frequently reported in weed biotypes resistant to ACCase-inhibiting herbicides is a resistant target site, ACCase. Non-target-site resistance mechanisms, such as enhanced metabolism of herbicide, have so far been rare.[34] However, increased metabolism of diclofop-methyl has been observed in one biotype of *L. rigidum*.[13] Where resistant weeds have at least two mechanisms of resistance, target-site-based resistance will most often be the most significant mechanism.[34] The resistant NAS 4 biotype not only has an insensitive target ACCase, albeit not sufficient to confer the high level of resistance to diclofop-methyl observed at the whole-plant level, but it also has an increased capacity to metabolise diclofop-methyl. It is likely that these two mechanisms of resistance are present in each individual of the resistant NAS 4 population (Table 3). Together, these two mechanism enable the resistant NAS 4 plants to withstand very high rates of herbicide.

Multiple mechanisms of resistance to the same herbicide have been reported in biotypes of *L. rigidum*, an obligate out-crossing species.[35] For example, *L. rigidum* biotype SLR 31 has an enhanced metabolism of diclofop acid and a resistant ACCase.[13,36] These two mechanisms may occur in different plants with only 12% of this biotype containing the insensitive ACCase.[36] It was noted that herbicide treatment of a large, polymorphic population results in the survival of individuals that possess one or more (different) mechanisms conferring the ability to withstand the herbicide application.[37] With obligate out-crossing species like *L. rigidum*, there is gene flow among the survivors, resulting in exchange of different resistance genes and their accumulation in the next generation. Therefore, depending upon genetic variation, the size of population exposed to herbicide selection, and the ability and efficiency of cross-pollination, there can be enrichment of a number of different resistance mechanisms.[37] *A. fatua* and *A. sterilis* are self-pollinated species with only a small degree of out-crossing.[38,39] It is, therefore, an indication of biological diversity that two mechanisms of resistance, both contributing to resistance, have appeared in a single biotype of *A. sterilis*. Resistance to diclofop-methyl endowed by a resistant ACCase in *A. sterilis*, *L. rigidum* and *L. multiflorum* is conferred by a single, nuclear-encoded, incompletely dominant gene.[40–42] In the case of *A. sterilis* biotype NAS 4, which has two mechanisms responsible for resistance, there must be more than one gene involved. Definite proof of the number of genes conferring resistance in biotype NAS 4 has yet to be obtained.

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